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BBI

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Mechanismy ovlivnění imunitního systému mikroby
Modulation of the Immune System by Microbiota

Bakalářská práce

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Abstract

Human microbiota is a collection of microorganisms colonising human´s body. The most important group of microbiota is represented by intestinal bacteria. Microbiota communicates with its host via intestinal mucosa and gut associated lymphoid tissues. Communication is mediated by microbial modulatory molecules such as short-chain fatty acids, lipopolysaccharide, polysaccharide A or immunomodulatory proteins. These molecules shape host´s physiology and particularly host´s immune system to accept close contact with hundreds of trillions of microorganisms. Disruption of this communication by altered microbial composition leads to diseases including inflammatory bowel disease, colorectal cancer, allergies, irritable bowel syndrome and others.

Keywords: microbiota, mucosal immunity, gut-associated lymphoid tissue (GALT), short-chain fatty acids, dysbiosis, inflammation

Abstrakt

Lidská mikrobiota je soubor mikroorganismů, které kolonizují naše tělo. Nejduležitější skupinu představují střevní bakterie. Mikrobiota komunikuje se svým hostem přes střevní sliznici a střevní slizniční imunitní systém. Komunikace je zprostředkována modulativními molekulami jako jsou mastné kyseliny s krátkým řetězcem, lipopolysacharidy, polysacharid A nebo imunomodulativní proteiny. Tyto molekuly ovlivňují fyziologii hostitele a obzvlášť jeho imunitní system tak, aby přijmul úzký kontakt se stovkami bilionů mikroorganismů. Porušení tohoto komunikačního systému změnou složení střevní mikrobioty vede k onemocněním jako jsou zánětlivé onemocnění střev, rakovina tlustého střeva, alergie, syndrom dráždivého tračníku a další.

Klíčová slova: mikrobiota, slizniční imunita, střevní mukózní lymfatický systém (GALT), mastné kyseliny s krátkým řetězcem, dysbióza, zánět

List of abbreviations

Antibiotics	ATB
Colorectal cancer/carcinoma	CRC
Dendritic cell	DC
Follicle-associated epithelium	FAE
G protein-coupled receptor	GPCR/GPR
Helper T cell	Th
Histon deacetylase	HDAC
Inflammatory bowel disease	IBD
Interleukin	IL
Intestinal epithelial cell	IEC
Irritable bowel syndrome	IBS
Lamina propria	LP
Lipopolysaccharide	LPS
Mesenteric lymph node	MLN
Microbial anti-inflammatory molecule	MAM
Microfold cell	M cell
Mucosa/Gut associated lymphoid tissue	MALT/GALT
Operational taxonomic unit	OTU
Pattern recognition receptor	PRR
Polysaccharide A	PSA
Prostaglandin E2	PGE_2
Regulatory T cell	Treg
Short-chain fatty acid	SCFA
Subepithelial dome	SED
T cell receptor	TCR
Toll-like receptor	TLR

Outline

1 Introduction

The human microbiota is a group of microorganisms occupying the human body. It is composed of archaea, viruses, fungi and bacteria (Aggarwala, Liang, & Bushman, 2017; Hoffmann et al., 2013; Qin et al., 2010). These organisms colonise various different niches including respiratory system, urogenital tract, oral and nasal cavities, skin and gastrointestinal tract each with signature composition (Huttenhower et al., 2012). The abundance of microbiota differs among niches but the overall quantity overtops human adult body cells 1.3 times if erythrocytes are included (Sender, Fuchs, & Milo, 2016). Human gut by itself contains as much as 100 trillion microorganisms (10^{13}-^{14}). Genome of gut microbiota alone contains more than 3 million genes which outnumbers the human genome more than 100-fold having under 25 thousand (Gill et al., 2006; Qin et al., 2010). A term microbiome is often used and it refers to the collection of all microbial genomes in the body. Notably terms microbiome and microbiota are often interchanged or used as synonyms.

Microbiota and it´s development reacts to environmental factors. The healthy composition of species thus differs remarkably among individuals. Interestingly the metabolic pathways remain relatively stable. The most significant habitat is the gastrointestinal tract which has the highest abundance of microbes. It consists of stomach, duodenum, jejunum, ileum, cecum, colon and appendix. The concentration of bacteria generally rises from stomach (10^2 bacteria/ml) to colon (10^{12} bacteria/ml). There are four main bacterial phyla in the GI tract each with various abundances: *Firmicutes* (49 %), *Bacteriodetes* (23 %), *Proteobacteria* (21 %) and *Actinobacteria* (5 %) (Frank et al., 2007; Zhang et al., 2014). For more detailed composition see Figure 1. There are 100 to 10000 times more anaerobes than aerobes in the GI tract with increasing anaerobic distal abundance. The upper tract features severe conditions of low pH, pancreatic secretion and bile thus it harbours fewer species. In distal segments the environment gets more moderate. Microbiota of the intestine and mainly colon is therefore the most significant for studies and for this thesis as well. It is defined by stool samples which contain 10^{11} – 10^{12} microbes/ml in which 70 % of bacteria colonises the colon in the long-term (Ley, Turnbaugh, Klein, & Gordon, 2006; Palmer, Bik, DiGiulio, Relman, & Brown, 2007).

The microbiota participates in many essential body functions including immune modulation, metabolism and neurologic processes (Hsiao et al., 2013; Turnbaugh et al., 2006; Willemsen, Koetsier, van Deventer, & van Tol, 2003). According to the nature of effects in anthropocentric view we divide microbiota into three groups: probiotics (beneficial), commensals (neutral) and pathogens (deleterious). However this division is potentially incorrect because the favourableness of the effect depends on the host ´s body state and current composition context. What effects microbiota has, how is the communication mediated and what are the causes of communication disruption are the main topics of this thesis.

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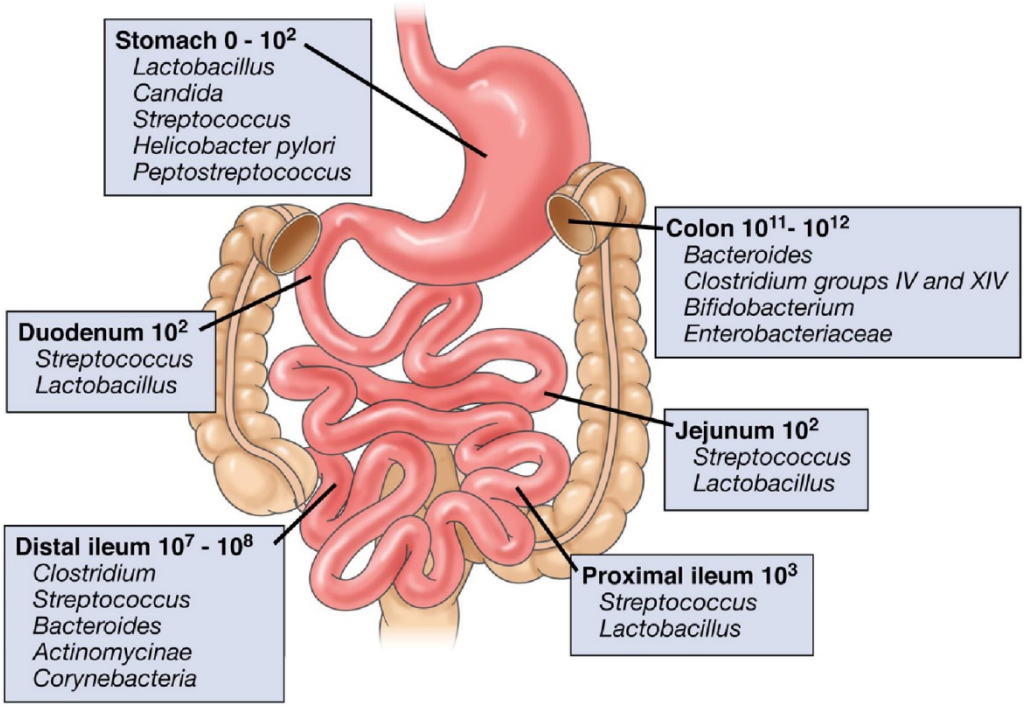

Figure 1: Composition and luminal concentration of dominant microbial species in various regions of gastrointestinal tract (Sartor, 2008)**.**

1.1 Factors shaping the human microbiota

As mentioned the composition of microbiota is heavily shaped by environmental factors. Genetic ancestry is not significant in the microbiota composition. The average heritability of composition is 1.9 %. Genetically unrelated individuals who share house-hold exhibited very similar composition whereas relatives who do not share household differed in composition remarkably (Palmer et al., 2007; Rothschild et al., 2018). Environmental factors which shape the microbiota are mainly birth delivery mode, diet and antibiotic (ATB) treatment. Humans are kept germ free throughout the whole ontogeny process. The colonisation starts with the birth. The first colonisers depend on the way of delivery. Via vaginal delivery the first microbes occupying the infant´s body are set by the composition of maternal vaginal microbiota. Thus species like *Lactobacillus* and *Bifidobacterium* exceed whereas caesarean section born infants resemble more skin-like composition of initial microbiota (Dominguez-Bello et al., 2010). Most of these differences gradually decrease. The impact of infant diet is also prominent. Breast-fed infants are colonised by more *Lactobacilli* and *Bifidobacteria* in comparison to formula-fed infants (Stark & Lee, 1982). Diet has major impact even in adulthood. In changes of fat and fibre ration the composition changes happen within 24 hours (Wu et al., 2011). Changes in higher taxa are noticeable in long-term diet changes. ATBs diminish level of bacterial diversity and conversely increase relative abundances of certain taxa. Most of these disturbances do not persist for long

however ATBs with broad activity like Clindamycin cause long-term effect. Disturbances after Clindamycin were clear even two years after treatment (Jernberg, Löfmark, Edlund, & Jansson, 2007). Furthermore heavy ATB use in first year of life is associated with higher inflammatory intestinal diseases incidence (Hviid, Svanström, & Frisch, 2011). Alternatively in mice a factor which strongly modulates the microbiota composition is stress (Aguilera, Vergara, & Martínez, 2013). If there are no major changes in mentioned factors adults keep fairly stable microbiota composition over time. In five years 60 % of the gut bacteria composition is retained. However Bacteroidetes and Actinobacteria are more stable than Firmicutes and Proteobacteria (Faith, Guruge, & Charbonneau, 2013).

1.2 How to study microbiota

Intestine offers very specific environmental conditions. Cultivation of intestinal microbiota is thus complicated if only because most of microbiota belongs to anaerobic species. A big step in defining microbial composition of the intestine was the introduction of metagenomics, metatranscriptomics and metabolomics (Pace, Stahl, Lane, & Olsen, 1986). They are used for identification of: operational taxonomic unit, transcription activities and metabolic abilities of bacteria. The process involves nucleic acid isolation from a selected sample (for example a stool sample) which is cloned into a vector and subsequently transformed into a host bacterium. Result is a suitable substrate for screening and for instance 16S rRNA identification (Venter et al., 2004). Human microbiome project defined the microbial composition by using metagenomics (Consortium, 2012). A useful technique in studies of microbiota is gnotobiology. Gnotobiology operates with either germ-free (GF) or germ-specific colonised animals. Animals in gnotobiological studies are bred in isolators for sterile environment. This allows us to see how animals react to certain conditions in lack of microbiota (Erny et al., 2015). Specific colonisation allows to colonise an animal with defined microbes which could be transplanted from a different animal or a disease affected animal (Turnbaugh et al., 2006). Alternatively there is a possibility co colonise an animal with single species and observe the effects on the host (Ivanov et al., 2009).

2 The mucosal immune system

2.1 Structure of mucosal immune system

Mucosal membranes serve many different functions from simple mechanical to homeostatic but primarily functions related to substrate transfer via secretion and absorption. Mucosal membranes cover oral and nasal cavities, respiratory system, urogenital system and digestive tract. Since mucosal main function is transfer, it is naturally used as a door to our body by pathogens (Eckmann, Kagnoff, & Fierer, 1993). In fact most pathogens infect human body via mucosal membranes. Mucosal membranes therefore need to keep a strong barrier function to prevent infection. However mucosal

membranes get into contact with many harmless antigens, where is no need to induct a defensive immune reaction. Such reaction would lead to diseases (Björkstén, Naaber, Sepp, & Mikelsaar, 2001). Mucosal membranes thus need to keep tolerance against these harmless antigens to prevent overreaction of immune system. These are the functions of the complex intestinal immune system.

Intestinal immune system consists of extracellular components and cellular components partially localised in mucosa associated lymphoid tissue (MALT). Extracellular components consist of physical and chemical environmental conditions. Cellular components consist of two groups: innate group of cells which are either intestinal epithelial cells or immune cells and adaptive group which is represented by effector antigen specific lymphocytes (Eckmann et al., 1993; Chang, Hao, Offermanns, & Medzhitov, 2014; Ivanov et al., 2009; McCormick, Colgan, & Delp-Archer, 1993). To understand the components entirely we first need to define the anatomy and physiology of intestinal mucosa and MALT.

2.1.1 Anatomy of the mucosal immune system

The intestinal mucosa covers approximately 250m^2. It is composed of a villous cell epithelial monolayer formed by intestinal epithelial cells (IECs) with several other specialised cell types placed along. Specialised cells in the epithelium include of goblet cells, Paneth cells, endocrine cells, stem cells and M cells. Each of these cell types serves a different function (Francisca et al., 2015; M. E. V. Johansson, 2012). Their apical membranes face towards the intestinal lumen. This side is covered by a layer of mucus. Basal membranes are bound to fibrous lamina propria (could be referred as subepithelial section) and face the opposite side. This epithelium binding crossover section could be referred as basement membrane. At the top of lamina propria are subepithelial fibroblasts. Lamina propria is interlaced with blood and lymphoid vessels which lead into each villus. Under lamina propria is a layer of muscularis mucosa and submucosa.

The anatomy of MALT slightly differs among mucosal organs. Our organ of interest is the intestine therefore we can specify mucosa associated lymphoid tissue to gut-associated lymphoid tissue (GALT). GALT is divided into organised tissue which represents the immunity induction site and scattered lymphoid cells which represent the effector site. Both are set under or in the intestinal epithelium and are in contact with the intestinal lumen. An important part of immunological processes effecting GALT is provided by a fraction of innate immunity cells which are often not included in GALT definition. It is composed of IECs and immune cells in lamina propria outside of organised tissue.

2.1.2 Physiology of gut-associated lymphoid tissue

Organised tissues consist of following cell types. The surface layer of cells contains columnar epithelial cells and microfold cells (M cells) collectively creating follicle associated epithelium (FAE).

FAE columnar epithelial cells feature less pronounced brush border and they do not secrete digestive enzymes in high volumes as digestion is not their main function. M cells are specialised enterocytes that lack microvilli and thick layer of mucus at the surface. Immediately beneath FAE are leukocytes including B lymphocytes, T lymphocytes, dendritic cells or macrophages. These leukocytes can be inserted into invaginated M cells for ultimate contact. Under FAE is more diffuse section of subepithelial dome (SED) with highest presence of migrating dendritic cells. SED covers the core section of lymphoid follicles which contains B lymphocyte and T lymphocyte areas (Kelsall & Strober, 1996). These follicles can be organised into macroscopic structures Peyers patches or into other types like cryptopatches in mice (Nochi, Denton, Wahl, & Garcia, 2013). These structures are connected to mesenteric lymph nodes (MLNs) via afferent lymph vessels. Scattered lymphoid cells lie in the epithelium and sub-epithelium of villus enterocytes. They are connected to the immune system via lymph and blood vessels. Leukocytes which occur in lamina propria of villous epithelium which do not belong to effector scattered lymphoid cells group include dendritic cells and macrophages (Soesatyo, Biewenga, Kraal, & Sminia, 1990). Dendritic cells serve an inductor function. Their dendrites lead through the epithelium into the intestinal lumen where they bind antigens (Rescigno et al., 2001). Every molecular or cellular item plays a role in the complex intestinal immune system whether it is creating a wall or promoting an immune response.

2.2 Functional process of intestinal mucosa immune system

2.2.1 The intestinal barrier function

Extracellular and cellular components establish collectively the intestinal barrier function. It´s main function is to restrict translocation of antigens and thus prevent infection of human body by pathogens. To access the intestinal cellular immune system an antigen or bacterium first needs to pass extracellular levels of intestinal barrier. It is the lumen content itself where the selection starts.

Lumen features a low pH, a wide variety of substances including pancreatic and bile juice, digestive enzymes, lipases, proteases, amylases, nucleases. Many microorganisms are not adapted to such conditions which leads to their cytolysis (Giannella, Broitman, & Zamcheck, 1972). Many antigen molecules are broken down. Next level of barrier function is the mucus layer at the top of the epithelium. Mucus is secreted by Goblet cells in a form of glycoproteins like mucin (M. E. V. Johansson, 2012). It creates a wall which protects the intestinal wall from luminal content and makes microbial adhesion more complicated. The outer layer of mucus contains antimicrobial peptides secreted by Paneth cells and secretory IgA secreted by plasmatic cells (antibody secreting stage of B cells). Paneth cells lie in the epithelial crypts and form small clusters. Antimicrobial peptides of Panteh cells are: α-defensins, CRS peptides, lysozyme C, phospholipase A_2 and ribonucleases. These peptides are secreted in granules and they diffuse from the crypt lumen to the outer mucosa layer (Meyer-Hoffert et al., 2008). Under the apical mucus layer lies a more dense layer of glycocalyx (Frey

et al., 1996). It has mostly protective role against luminal content. It also helps with absorption of nutrients. Glycocalyx is the second and last covering layer. It lies directly on the intestinal epithelium.

It is crucial to keep intestinal epithelial cells well bound together to prevent the possibility of paracellular transport of antigens. IECs are bound together via complexes of intercellular junctions including tight junctions, adherent junctions, desmosomes and gap junctions. Tight junctions seal the gap between epithelial cells, adherent junctions are connected to actin filaments of abutting cells, desmosomes connect the intermediate filaments and gap junctions allow passage of small water-soluble molecules. These structures regulate paracellular transport to maximal size of 50 Å. Their transmembrane protein of use is cadherin. On the other hand cell-matrix adherent junctions like hemidesmosomes anchor epithelial cells (actine filaments or intermediate filaments) to extracellular matrix (in the basal direction) via integrin proteins. The function of intracellular junctions is mainly to a form bond between all epithelial cells and to anchor them to the lamina propria (Boyle et al., 2008). Other features which take part in the barrier function are secretion of water by enterocytes and peristalsis of intestinal wall (Vantrappen, Janssens, Hellemans, & Ghoos, 1977). The final result of the intestinal barrier function is a decrease of: microbial attachment, interaction of microbial antigens with immune cells and the possibility of microbial translocation into the body.

Human microbiota was able to adapt to these tough conditions and creates a key part of barrier function called colonisation resistance. Potential pathogens (*enteropathogenic E. coli, Salmonella, Shigella, Vibrio* and others) have to compete with human microbiota for nutrients and adhesion sites. Such competition leaves small chance to pathogens considering microbiota´s advantageous relationship with host. Protective conditions include antimicrobial substances secretion by microbiota as well as the host or mucin secretion by host´s cells after a microbial stimulus. Lactic acid bacteria produce antimicrobial organic acids such as acetic acid or lactic acid as well as hydrogen dioxide, carbon dioxide or bacteriocins (Tasakis & Touraki, 2018). *Bifidobacteria* strains can directly inhibit pathogenic adhesion by blocking its adhesion receptors and thus avert epithelial infection (Bernet, Brassart, Neeser, & Servin, 1993). *Lactobacillus plantarum 299v* and *Lactobacillus rhamnosus* increased the mucin genes expression of IECs (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). Furthermore there is a form of cooperation among microbiota. *Lactobacillus GG* and *Lactobacillus bulgaricus* were able to enhance the adhesion of different bacterial strain *Bifidobacterium lactis* (Ouwehand, Isolauri, Kirjavainen, & To, 2000). This suggests a trend of chain effect in the intestinal microbiota composition.

2.2.2 GALT function

To induct an immune response whether it is going to be defensive or tolerant we first need a contact between an antigen and a cell. The process starts with transcytose of antigen via M cells to antigen presenting (APC) dendritic cells (DC) lying on the edge of dome section cooped into these M cells.

DCs than present the antigen to B and T lymphocytes in lymphoid follicles (Kelsall & Strober, 1996). These primed lymphocytes afterwards migrate to MLNs where they differentiate to mature cells. Than they migrate via blood vessels back to the mucosa using integrins α4β7 which bind endothelial cell adhesion molecule-1 (MadCAM-1) a homing receptor which leads them specifically to intestinal mucosal tissue (Berlin et al., 1993). These lymphocytes now being matured effector cells than find place outside of organised tissues in villi and crypts of intestinal epithelium.

B cells remain in lamina propria. They differentiate into final plasmatic state and produce large amounts of secretory IgA. $CD4^+$ T lymphocytes are distributed through lamina propria of villous intestinal epithelium. They differentiate into regulatory (Treg) and helper (Th) cell types. Tregs produce cytokines IFN-γ and IL-10 (Braunstein, Qiao, Autschbach, Schurmann, & Meuer, 1997; Carol et al., 1998). Their main function is regulation of local immune response and tolerance induction. Tolerance could be maintained by antigen-presenting villous IECs by keeping the activity and survival of these $CD4^+$ lymphocytes (Westendorf et al., 2009). Most $CD8^+$ T lymphocytes with cytoxic potential travel to intestinal epithelium, 40% remain in sub-epithelial section (lamina propria). The intraepithelial $CD8^+$ lymphocytes use their cytoxic activity to keep turnover of damaged or stressed enterocytes and to protect us against intestinal infections using proinflamatory cytokines (Guy-Grand, Cerf-Bensussan, Malissen, & Malassis-Seris, 1991). On the other hand they have ability to supress inflammation using IL-10 and TGF-β and help enterocytes to regenerate (Miller, Lider, Roberts, Sporn, & Weiner, 1992). Effector T-lymphoytes in intestinal mucosa in general could also help intestinal B cells to produce IgA by positive stimulation. Partially they could also serve as memory immune cells (Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999).

An alternative pathway involves transfer of antigen through intestinal epithelium via lamina propria dendritic cells which than travel to MLNs using afferent lymphoid vessels and prime naive lymphocytes (unlike previous option where naive lymphocytes were primed in lymphoid follicles) (Rescigno et al., 2001). These MLN primed lymphocytes than migrate either as effector cells to villous membranes through efferent lymph vessels and bloodstream or exit the bloodstream and spread via peripheral immune system to create a systemic distribution. This creates a systemic presence of differenced specific antigen tolerant CD^{4+} lymphocytes. Last option is that free antigen travels from villus lamina propria through afferent blood vessels to peripheral lymph nodes (unlike previous options where antigen was transported by DCs to MLNs) (Bell et al., 2001). This option can thus leads to systemic distribution as well. Such process of orally administered antigen which leads to systemic toleration is called oral toleration. Most of mentioned effects have been studied on mice, rats and in vitro. The whole process has been elegantly reviewed (Fig. 2) (Mowat, 2003).

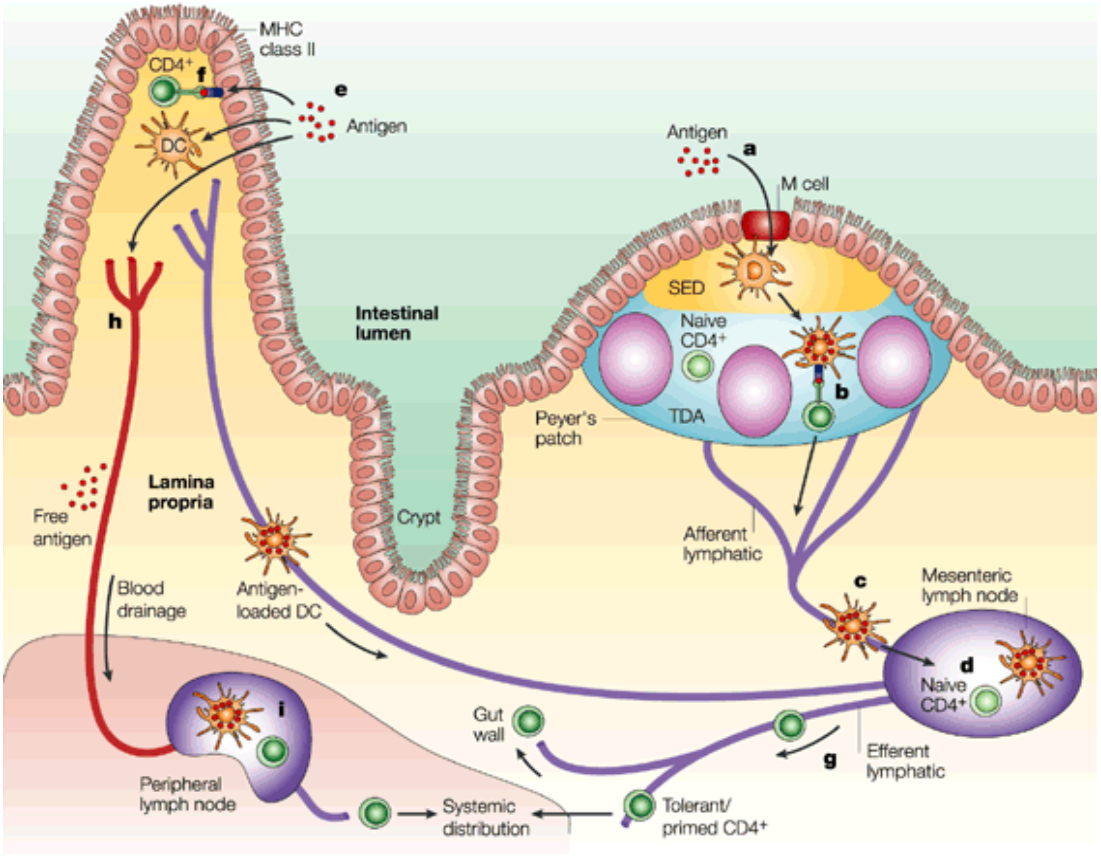

Figure 2: Antigen uptake and recognition by CD4+ T cells in the intestine (Mowat, 2003)**.**

2.2.3 Differentiation between pathogen and commensal

Whether the response to antigen is going to be tolerant or inflammatory depends on the character of antigen. How does the immune system tell if the antigen is potentially pathogenic or harmless and what is the character of each response is going to be the next topic.

The processing of antigen and presentation is mediated by DCs and IECs (Westendorf et al., 2009). Microbial antigens are generally referred as microorganism-associated molecular patterns (MAMPs). Corresponding antagonist receptors are pattern recognition receptors (PRRs). MAMPs are mostly molecules from the surface of bacteria including peptidoglycan (PG), call-wall associated polysaccharides (CPS), glycoproteins, lipoproteins, secreted proteins, fimbriae, flagella as well as wall teichoic acids and lipoteichoic acids in terms of gram-positive bacteria and lipopolysaccharides (LPS) in terms of gram-negative bacteria. Although MAMPs share similar basic structure, there are variable sections of these molecules that characterise their holder. Variations can be in glycosylation of teichoic acids, structure of CPS or Lipid A section of LPS (Hajjar, Ernst, Tsai, Wilson, & Miller, 2002; Xia et al., 2010). Perhaps the most important are toll-like receptors (TLRs). There are specific types of TLRs to certain MAMPs. After the binding of specific ligand TLRs induce a signal cascade that leads to secretion of cytokines (for instance IL-1, IL-6, TNF, IL-8), anti-apoptosis signals, tight junctions

stimulation, defensin secretion or inflammatory response. TLRs are expressed by immune cells and IECs. (Otte, Cario, & Podolsky, 2004) However there are other PRRs that take a part as well. CD14s bind lipopolysaccharides, C-lectin type receptors bind microbial saccharides and cytosolic NOD-like receptors recognise mainly peptidoglycan fragments after phagocytosis and danger-associated molecular patterns released from hosts damaged cells (which leads to secretion of IL-1 and IL-8). Information acquired from receptor overview (Hořejší, Bartůňková, Brdička, & Špíšek, 2017).

The main reasons why commensals do not trigger an inflammatory response is because commensals lack virulence ligands that pathogenic bacteria have and use against host cells. For example the pathogenic P fimbriae expressing E. coli utilise P pilus type for adhesion. These fimbriae bind glycosphingolipid recognition receptors on epithelial cells which trigger TLR4 signals and specific adaptor proteins (TRIF/TRAM pathway) which lead to neutrophils recruitment and local mucosal inflammation (Fischer, Yamamoto, Akira, Beutler, & Svanborg, 2006). Furthermore the presence of toxins diminish several suppressive pathways of monocytes (Cox et al., 2009).

Next principle that works in favour of commensal toleration is suppression of host´s inflammatory response by commensal bacteria. Specific signalling suppressive pathways will be described in following chapter. There are two main suppressive pathways. First involves decreased activity of transcription factor NF-κB and the second involves induction of Foxp3+ regulatory T cells which secrete anti-inflammatory cytokines. Foxp3 is a transcription factor with major impact on Treg differentiation. NF-κB is responsible for expression of inflammatory genes. It is composed of two subunits: p50 and ReIA. It´s inhibitor which blocks nucleus translocation is IκB-α. An anaerobic bacterium *Bacteroides thetaiotaomicron* enhances the export of NF-κB subunit ReIA from nucleus via PPAR-γ-dependent pathway making it non-functional (Kelly et al., 2004). Another study depicts nonvirulent Salmonella strains that block IκB-α ubiquitination. NF-κB is thus unable to translocate to the nucleus (Neish et al., 2000). Some commensal bacteria thus supress inflammatory response and pro-inflammatory cytokine secretion which facilitates colonisation of the host.

Plasmatic cells play an important role as well. They produce immunoglobulins which are transported via process of transcytose. Antibodies produced in the sub-epithelium are bound to the poly-Ig-receptor of epithelial cells. Than are the imunoglobulins transported to the intestinal lumen with a small segment of the receptor kept intact. The segment is called secretion component and it prevents the antibody from luminal proteases. Plasmatic intestinal cells produce mainly secretory IgA (sIgA) but IgM occurs as well. sIgA has mainly neutralizing function towards commensals and pathogens. It does not recruit the complement, it mostly blocks the bacterial adhesion to the epithelial cells. This prevents infection and chronic inflammation but it does not interfere with the commensal bacterial life cycle (Boullier et al., 2009). sIgA2 is thus targeted against commensal which helps to set the symbiosis straight (Weltinz, Lucia-Jandris, Michetti, & Fields, 1989).

According to the nature of the microorganism immune system can either set up an inflammatory response or a tolerant response. Inflammatory response starts as mentioned with a local inflammation. Epithelial cells and macrophages in lamina propria secrete pro-inflammatory cytokines like IL-1, TNF-α, IL-6 and IL-8 (Komatsu et al., 2009). This leads to DCs maturation. DCs absorb pathogenic antigens, increase their MHC and costimulatory molecules expression and migrate to MLNs (lamina propria DCs) or Peyers patches (SED located DCs) where they prime naïve T lymphocytes. Priming happens via T cell receptors, co-stimulation of CD28 rec by CD86 ligand on DC as well as stimulation of cytokine receptors by DC secreted IL-12. This combination leads to preferential differentiation of Th1 cells. These maturated cells than migrate to mucosa to further the inflammation. They secrete INF-γ which activates local macrophages to prevent intracellular parasitism (with CD40L co-stimulation) and enhances cytokine IL-1, IL-2 and TNF secretion (Hsieh et al., 1993). They overall stimulate local inflammation to block the infection. Alternatively a co-stimulation by IL-4 is preferred which leads to Th2 differentiation (Serre et al., 2010). Th2 cells stimulate antibody secretion by B lymphocytes and support barrier function. Th1 and Th2 tend to supress each others activities. The result depends on IL-12 and IL-4 concentration.

On the other hand if there is no primer inflammation caused by cytokine secretion after a virulent antigen stimulus than response is different. Prostaglandin E_2 (PGE_2) is secreted by local macrophages and TGF-β is secreted by epithelial cells (and possibly IL-10) (Newberry, McDonough, Stenson, & Lorenz, 2001). With this stimulation and commensal antigen absorption, maturated DCs migrate to MLNs or Peyers patches to prime T lymphocytes (with commensal antigen presented on MHC II). What is different is the selection of cytokine co-stimulation being IL-10 and TGF-β as well as stimulation of inhibition receptor CTLA4 on T cells. T cells mature to either effector induced T regulatory cells which secrete IFN-γ and IL-10 or Th3 cells which secrete TGF-β (Harizi, Juzan, Pitard, Moreau, & Gualde, 2002; Miller et al., 1992). Both cell types than migrate back to mucosa or undergo process of systemic redistribution. The result is promotion of tolerance to a specific commensal antigen.

The whole system balances in a dynamic equilibrium of physiological inflammation. Inflammatory responses occur. They are drove by adaptive and innate branch of immune system. The key role play effector T regulatory cells which supress inflammatory response to commensal antigens and keep the response balanced.

3 Modulatory molecules

Intestinal microbiota communicates with its host via many types of molecules. These molecules are capable of physiology modulation and particularly immune system modulation. This continuous stimulation by microbial product is crucial for hosts development and function of immune system.

Deficiency in this stimulation abrogates human immune homeostasis and leads to diseases. In this chapter will be described effects of several microbial modulatory molecules.

Modulatory molecules are bacterial metabolites. They could be divided into two groups. First are products of catabolism and second are products of anabolism. The former group involves metabolites produced by many different metabolic pathways, often unique for a bacterial species, which extract energy. Whereas anabolic products are either synthesized structural components or secreted proteins. Structural components construct cellular wall, capsule and other structures and they continuously detach from their carriers and dilute into the intestinal lumen. The result is corresponding concentration of each molecule in the intestinal lumen (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). Portion of these molecules is utilised by other bacteria some are broken down or excreted but significant portion dilutes to the intestinal epithelium. Most of them are transported by epithelial cells, M cells or dendritic cells to be processed in lamina propria or organised tissues and take local or systemic effect. Alternatively molecules take effect directly on epithelial cells. The immune reactivity toward these molecules has probably evolved as a protective measure.

3.1 Lipopolysaccharides

Lipopolysacharides are surface molecules of Gramm-negative bacteria. They consist of lipid A which anchors them to the outer membrane and polysaccharide section. Polysaccharide section consists of inner core, outer core and multiple repeating fragment called O antigen. LPS promote secretion of inflammatory cytokines. They are bound by TLR4 with a help of couple co-receptors (Visintin et al., 2001). Lipopolysaccharide binding protein extracts LPS from the bacterial outer membrane. CD14 delivers the lipid A fraction to MD2 protein which associates with TLR4. That causes dimerization of TLR4 and sets up a signal. TLR4 signalling functions in two pathways. First is the TIRAP-MyD88 pathway which activates NF-κB via IKK complex (Erridge, Pridmore, Eley, Stewart, & Poxton, 2004). Early NF-κB activation leads to proinflammatory cytokine expression such as IL-12. Second pathway called TRIF-TRAM activates interferon regulatory factor-3, a transcription factor which activates expression of type 1 interferons and TNF-α. TNF-α as a TLR4 agonist that furthers the NF-κB activation. A number of LPSs which signal through TLR4 are considered to be the most active endotoxin agents. The intensity of cellular response is dependent on the character of the LPS. For instance the intensity of TNF-α secretion by monocytes varied widely among different species LPS stimuli (Pridmore et al., 2003). LPSs of several G^- bacterial species have been proven to be ligands of TLR2. TLR2 signalling leads to NF-κB activation too however the cellular effects and subsequent biological activity effects are weaker (Erridge et al., 2004). TLR2 signalling could be thus beneficial in contact with commensal bacteria.

The expression of TLR4 differs among tissues and cell types. TLR4s are highly expressed by monocytes, macrophages and granulocytes as well as splenic cells. DCs express TLR4 in lower

numbers and in immature state only (LPS-TLR4 signalling is a DC activating pathway) (Visintin et al., 2001). Whereas other mentioned leukocytes enhanced their TLR4 expression after agonist exposure (Muzio et al., 2000). Intestinal and endothelial cells express TLR4s in lower numbers than monocytes or granulocytes. Expression on IECs has to be supressed to prevent chronic inflammation. IECs thus downregulate their TLR4 and MD-2 expression. Their overall responsiveness to LPS and activation of NF-κB is therefor much lower than a monocyte response (Abreu et al., 2001). Intestinal alkaline phosphatase play important role in decreasing possibility of IEC inflammatory response as well. They dephosphorylate LPSs turning them into non-toxic form. Dephosphorylated LPSs were shown to be non-toxic in zebrafish studies and their TLR as well as TNF signalling was decreased. These enzymes are localised mostly on the apical membrane limiting their detoxifying abilities to the intestinal lumen only (Bates, Akerlund, Mittge, & Guillemin, 2007).

The structure of LPS differs among bacterial species and so differs the cellular response. As mentioned TLRs bind the lipid A fragment of LPS. Differences in lipid A structure which are suggested to determine whether the signalling will be mediated via TLR4 or TLR2 are following: glucosamine phosphorylation, and acyl chains. Acyl chains vary in number, length, substituents, saturation or branching. For instance hexa-acetylated LPS strain of *Pseudomonas aeruginosa* with pathogenic properties is well recognised by TLR4 and triggers a substantive inflammatory response. *Pseudomonas aeruginosa* strain lacking pathogenic adaptation has penta-acetylated LPS. This strain does not trigger such inflammatory response (Hajjar et al., 2002). Even though LPS is a substance with endotoxic properties human body has developed mechanisms supressing it´s toxic and inflammatory effects. The response thus varies according to the structure of LPS and signalling pathway.

3.2 Short-chain fatty acids

Short-chain fatty acids (SCFAs) are products of catabolism. Microbiota utilise insoluble plant fibre from food to produce SCFAs in process of fermentation (Macfarlane & Macfarlane, 2006). Insoluble plant fibre is not fully digested by humans because we lack enzymes for breakdown of these molecules (Slavin, Brauer, & Marlett, 1981). However bacteria offer a wide range of membrane bound enzymes capable of breaking these polysaccharides down. SCFAs are essential microbe-host interaction molecules with an energetic source and signalling potential. Such signalling cascades end up modulating hosts physiology of immune or nervous system (Block, Rawat, Brosgart, & Francisco, 2015). This communication thus determines the possibility of several diseases. The concentration of insoluble plant fibre depends on host diet (Haenen et al., 2013). SCFAs communication is a process where diet composition modulates hosts health via essential microbial activities.

3.2.1 Production

Most of non-digestible molecules are plant cell-wall components such as cellulose, hemicellulose xylans (xyloglucans and glucoarabinoxylans), pectins or other oligosaccharides, storage saccharides (inulin) and resistant starch that evaded small intestine digestion (Chassard, Goumy, Leclerc, Del'homme, & Bernalier-Donadille, 2007; Tolonen et al., 2011). These molecules could be broken down by specialised bacteria. Polysaccharide-degrading bacteria species belong to phyla *Bacteroides, Firmicutes and Actinobacteria*. Typical cellulolytic commensals are *Rumnicoccus*, a *Clostridia* member or *Enterococcus*, a member of *Lactobacillales* (Montgomery, 1988; Robert & Bernalier-Donadille, 2003; Salyers, Vercellotti, West, & Wilkins, 1977). It is important to keep in mind that the microbial mediated breakdown of plant polysaccharides like SCFA fermentation is not a process done by one bacterial species. It is composed of chain of reaction done by various species. It is characterised by a rich microbial cooperation and cross-feeding. Cross-feeding is a competitive utilisation of products of primary degraders by other bacterial species (in this case secondary fermentation) (Freilich et al., 2011; Kato, Haruta, Cui, Ishii, & Igarashi, 2005).

The process starts with cellulolytic bacteria (an example of primary degrader). They use their enzymes to cleave plant cell wall components like pectins and hemicellulose links between the cellulose fibrils or cellulose itself. Most of cellulolytic bacteria use Cellulosomes. Cellulosom is a complex of enzymes anchored to the bacterial surface. It consists of main scaffoldin subunit which is bound to the bacterial surface by anchoring protein. Scaffoldin features a cellulose-specifis carbohydrate-binding molecule (which targets and binds the enzyme complex to cellulose) and cohesin modules which bind all enzymatic subunits that obtain specific cleavage activities like cellulase, xylanase, glucanase, hemicellulase or pectatase activities (Murashima, Kosugi, & Doi, 2002). The result is cleavage of cell wall polysaccharides into oligosaccharides. Oligosaccharides are transferred via ABC transporters into the bacterial cytosol where further metabolic processes continue. Hydrolysation is followed either by production of gasses which can be further oxidised by other species or by SCFA fermentation. Main SCFAs capable of modulation hosts physiology are acetate, propionate and butyrate (Kobayashi et al., 2017). The concentration of SCFAs in intestinal lumen ranges from 70 to 140 mM in ration 3:1:1 acetate to butyrate to propionate (Cummings et al., 1987). They are transported through apical membrane of epithelial cells and partially utilised directly in colonocytes as a source of energy. In fact SCFAs create up to 70% of their energy sources via oxidation to ketone bodies and CO_2 (Roediger, 1982). The remaining portion is transported through basolateral membrane by and enters sub-epithelial region making its way into subepithelial region to immune cells and into blood vessels. The final SCFA concentration in peripheral blood is only over 100 μM for acetate, around 5 μM for propionate and around 3 μM for butyrate (Cummings et al., 1987).

3.2.2 Effects

SCFAs modulate hosts physiology via two major signalling pathways. They interact with G protein coupled receptors (GPCRs or GPRs) or they function as histone deacetylase (HDAC) inhibitors. Signal cascades induced by SCFA bound to GPCRs effects cells differently according to the tissue and receptor type. HDAC inhibition can be either direct or indirect via GPCRs. GPCR signalling and HDAC inhibition are associated mainly with anti-inflammatory and tolerogenic effects. There are immune modulating processes dependent on GPCRs and there are immune modulating processes GPCR independent. Both of these principles are crucial for local homeostasis of the intestine and systemic homeostasis as well.

3.2.2.1 Histon deacetylase inhibition

HDACs are enzymes which promote deacetylation of histone N terminus. Histones with acetylated N-terminuses form relaxed euchromatine available for transcription. Deacetylated histones form denser heterochromatin which is unavailable for transcription factors. Thus inhibition of HDACs results in acetylated relaxed euchromatin form of DNA which actively expresses genes. However some HDACs are capable of deacetylation of multiple protein substrates like transcription factors as well (Chen, Wolfgang, Verdin, & Greene, 2001). Acetylation of transcription factors alters their activities. SCFA mediated HDAC inhibition promotes anti-inflamatory effects. That happens mostly via decreased transcription of proinflamatory genes thus decreased secretion of according cytokines via increased transcription of inhibitors (Vinolo et al., 2011). An important attribute of this principle is selective bioactivity. Inhibition of HDACs is specific which subsequently leads to either transcription of specific genes or activity modulation of a specific protein. The HDAC inhibition potential is a characteristic of all SCFAs however butyrate is the most potent. Most SCFAs show inhibition activities with concentration over 10mM, butyrate is effective at 2mM (Waldecker, Kautenburger, Daumann, Busch, & Schrenk, 2008). These findings also suggest that effective direct inhibition of HDACs happens mainly locally in the intestine. Peripherally may be preferred GPCR signalling.

Butyrate activates transcription of genes responsible for inhibition complexes forming in macrophages. These complexes inhibit expression of specific proinflamatory genes coding IL-6, IL-12 or NO secretion (Chang et al., 2014). Propionate and butyrate enter DCs via SLC5a8 transporter and inhibit HDAC 1 and HDAC 3. This inhibition results in block of DCs differentiation. This block of differentiation is from 75% to 40% dependent on mentioned transporter supporting the theory that this could be another GPCR independent HDAC inhibition (Singh et al., 2010). Neutrophils in ex vivo and in vivo studies decreased their inflammatory cytokine TNF-α and CINC-2αβ production after stimulation with propionate and butyrate (Vinolo et al., 2011). This process involves HDAC and NF-κB inhibition. HDAC inhibition likely affects other proteins which modulate activity of inflammation genes transcription factor NF-κB. Activity of NF-κB subunit ReIA is modulated by acetylation. Acetylation of this subunit weakens its interaction with the factor complex. HDAC 3 serves function

of ReIA deacetylation. With inhibition of HDAC 3 the NF-κB is incomplete thus the transcription of inflammatory cytokines is decreased (Chen et al., 2001).

HDAC inhibition has been also proven to stimulate Treg production and suppressive functions via modulating transcription factor Foxp3. Inhibition of HDAC 9 caused increased histone acetylation and subsequent expression of Foxp3. However it also increased acetylation of lysines on Foxp3 catalytic domain increasing its promoter binding activity. Result is enhanced expression of Treg genes. HDAC 9 inhibition increased numbers of $Foxp3^+$ cells in spleen and thymus by almost twofold. HDAC 9 inhibited Tregs are two to threefold more effective in supressing T-cell proliferation partly via IL-10 (Tao et al., 2007). This study used for inhibition trichostation-A. Mentioned SCFAs usually show a lower potency. Since direct HDCA inhibition is very concentration dependent and only mM concentrations are sufficient enough to inhibit HDACs effectively (Waldecker et al., 2008). SCFAs thus stimulate Treg function via GPCRs mediated signalling as well.

3.2.2.2 G protein-coupled receptor signalling

SCFAs are unique ligands of G protein coupled receptors 41,43 and 109A. GPCRs 41 are more widely expressed across different tissues. They occur in brain, spleen, intestine, lungs, bone marrow but mainly in adipose tissue. On the other hand GPCRs 43 are expressed mainly by immune cells and hematopoietic tissue (bone marrow and spleen) (Brown et al., 2003; Cox et al., 2009). GPCR 109A is expressed in intestinal and immune cells (Singh et al., 2010). Binding of ligand sets up a signal cascade which modulates cells physiology in various ways. This communication is essential for immunity homeostasis since mentioned GPCR deficient mice show increase of intestinal inflammation and diseases like colitis, arthritis and asthma and colon cancer (Chang et al., 2014).

GPR 109A has two agonists (ligands). First is niacin and second is butyrate. Butyrate´s bond induces GPR 109A signalling which has been proven to promote anti-inflammatory effects. Stimulation of macrophages and DCs with butyrate increased their anti-inflammatory molecules expression such as IL-10 and AldhA1. This led DCs to favour priming of naïve lymphocytes to Treg and IL-10 secreting cell types. GPR 109A is encoded by Niacr1 gene. Knockout of this gene caused increased intestinal inflammation and increased risk of colon cancer in mice (Maslowski et al., 2009; Singh et al., 2014).

GPCR 43 binds all three mentioned SCFAs. It is encoded by Ffar gene. Mice with Ffar knock out increased concentration of inflammatory mediators such as IL-17A, IL-6, IL-1α and IFNγ in colon (Maslowski et al., 2009). This led to increased neutrophil recruitment thus increased inflammation. And vice versa if there was an inflammation in colon, stimulation of wild-type mice with SCFAs eased off the inflammation and reduced the expression of proinflammatory receptors (C5aR, CXR2) on immune cells. GPR 43 has also been linked to modulation of monocytes by SCFAs. SCFA stimulation increased their PGE_2 production (Cox et al., 2009). PGE_2 limits T cell activation by DCs (Andrew J. Wiemer, Subramanya Hegde, Jenny E. Gumperz, 2012). However this effect was

diminished when pertussis toxin was present. SCFAs also inhibit monocyte chemotactic protein-1 (MCP-1) secretion which is responsible for monocyte migration towards inflammation (Ajuebor et al., 1998; Cox et al., 2009). However SCFAs also inhibited LPS-induced IL-10 production in monocytes showing a specific cell-type dependent SCFA influence (Cox et al., 2009). Furthermore SCFAs inhibited LPS induced secretion of inflammatory cytokines TNF-α and IFN-γ in peripheral blood mononuclear cells (lymphocytes, monocytes and DCs) (Maslowski et al., 2009). Since such oriented studies are still in early stages, the molecular background of signalling and cellular effect is lacking. Some of SCFA effects are still not linked with concrete molecular pathways.

3.2.2.3 Effects with undefined signalling pathways

As suggested SCFAs inhibit immune cell recruitment and migration towards inflammation. This principle is modulated via endothelial cell as well. Butyrate inhibited TNF-α induced expression of vascular cell adhesion protein-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). These two molecules are crucial for adhesion of lymphocytes, monocytes, eosinophils and basophils to the endothelium and subsequent migration into tissues. Their expression is induced by IL-1, TNF-α and IFN-γ if there is a local inflammation. Treatment with butyrate decreased IL-1β-induced ICAM-1 and VCAM-1 expression by endothelial cells by almost 80% and respectively by 37% for TNF-α inducted expression (Zapolska-Downar, Siennicka, Kaczmarczyk, Kołodziej, & Naruszewicz, 2004). Decrease is mediated via inhibition of NF-κB activity. Other principle of modulated neutrophil migration depicts decreased IL-8 secretion. A line of IECs decreased its IL-1β induced IL-8 secretion after treatment with butyrate. IL-8 is a neutrophil chemotactic factor which induces neutrophil migration towards inflammation (Böcker et al., 2003).

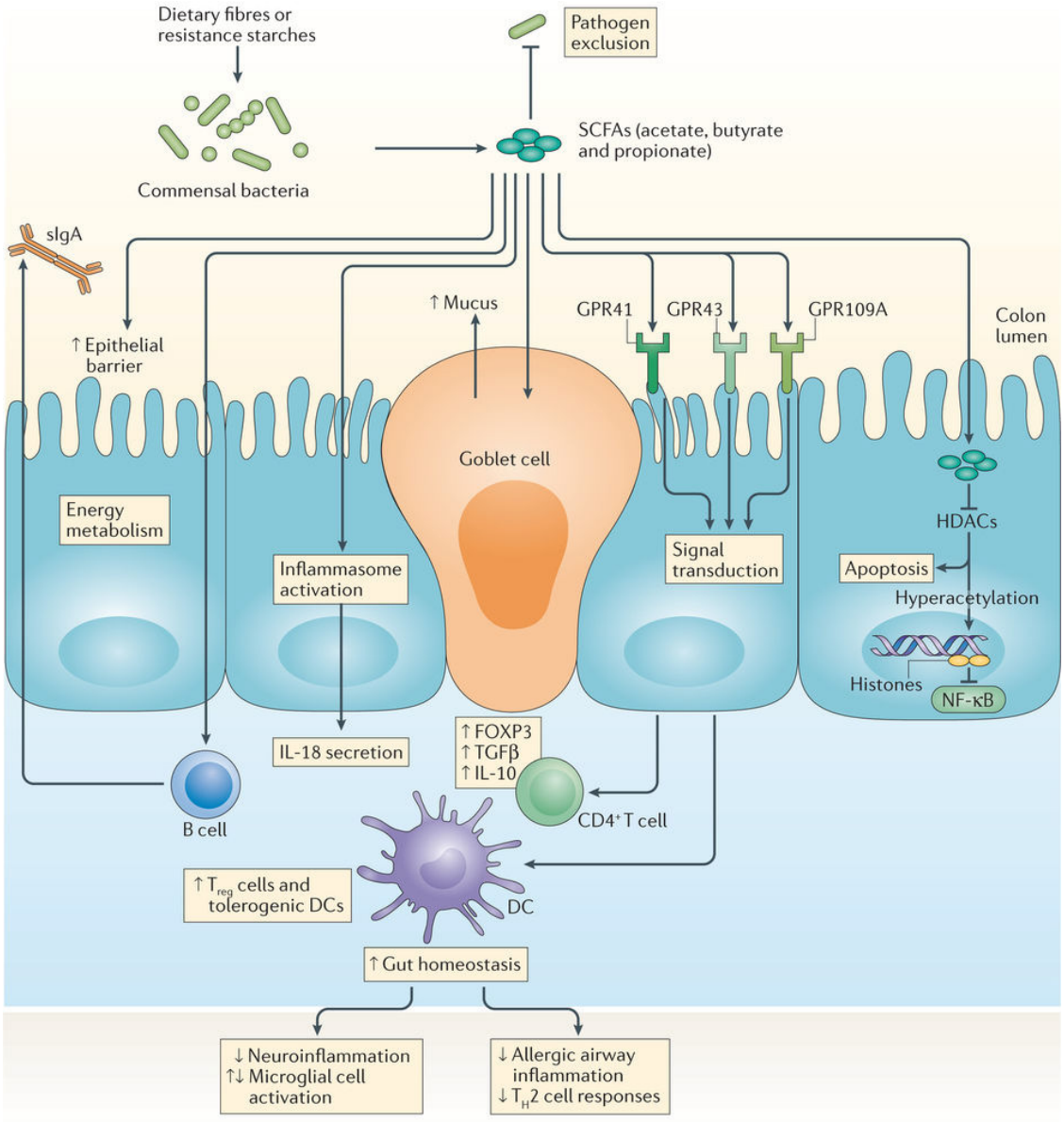

Figure 3: SCFAs, GPCRs, host physiology and immunity (Rooks & Garrett, 2016)**.**
Figure depicts described mechanisms of SCFAs including GPR signalling and HDAC inhibition. Notably signalling to immune cells happens similarly as depicted to IECs. Allergic reaction will be discussed in the last chapter.

However SCFAs modulate stimulate the intestinal barrier integrity as well. Stimulation with SCFA increased prostaglandin secretion by sub-epithelial fibroblasts which led to higher MUC-2 gene expression in goblet cells. These cells thus enhanced mucin secretion (Willemsen et al., 2003). Butyrate has been proven to decrease paracellular transport by facilitation of tight junction assembly in IECs and endothelial cells (Miyoshi, Usami, & Ohata, 2008; Peng, Li, Green, Holzman, & Lin, 2009). Assembly of intestinal tight junctions is enhanced via butyrate stimulated increase of AMP-activated protein kinase activity. Butyrate effects on IECs have been described even within the cell cycle.

Butyrate inducts apoptosis of tumorous proliferating cells in S phase and keeps intestinal cells in G0 and G1 phase (Qiu, Ma, Yang, Wang, & Jiang, 2017).

The effect of SCFAs on cellular immune system exceeds to central nervous system (CNS). Microglia function as macrophages in nervous system. They collectively sustain homeostasis of nervous system via scavenging of dying cells, pathogens and local molecules. The function of SCFAs in nervous system differs from previous examples. They promote maturation and active function of microglia in CNS. This modulated morphology enhances resistance against bacterial and viral challenge. Studies of germ free mice showed juvenile-like phenotype of microglia. A continuous SCFA input is thus needed to keep microglia in maturated state (Erny et al., 2015). Effects of SCFAs are summarised in Figure 3.

3.3 Polysacharide A

Polysaccharide A (PSA) is one of eight polysaccharides that confect capsule of commensal grammnegative anaerobic bacterium *Bacteroides fragilis*. PSA is the most expressed out of the eight polysaccharides. It is crucial for *B. fragilis* tissue associated colonisation of the colon, more specifically colonic crypts (its niche). PSA has several hundred oligosaccharide repeating units with free carboxyl, phosphate and amino units which carry positive and negative charges. These charges create zwitterion (dipolar ion) character of PSA which is possibly a key attribute for polysaccharide presentation and TCR recognition (Johnson, Jones, & Cobb, 2015). PSA is a presentable antigen and a ligand of TLR2 with immunomodulatory properties. Modulation of immunity by PSA involves innate and adaptive branch of immunity as well as specific TCRs and multi-ligand TLRs.

PSA is released by *B. fragilis* in outer mucus layer of the colon. Subsequently it is transported through the epithelium and partially taken up by subepithelial plasmacytoid DCs (pDCs), a line of dendritic cells with specific characteristic different from conventional DCs. pDCs than process the antigen and present it on their MHC II. An alternative way is direct signalling through TLR2 on DCs and CD4+ cells. Presentation of PSA on MHC II requires CD86 and ICLOSL expression on DCs and TCRβ and CDR3 recognition receptors and coreceptors on CD4+ T lymphocyte. This signalling leads to clonal expansion of antigen specific effector memory CD+4 cells. These cells are Foxp3+ Tregs which secrete IL-10 (Dasgupta, Erturk-Hasdemir, Ochoa-Reparaz, Reinecker, & Kasper, 2014). Direct signalling of PSA through TLR2 happens either on DCs which subsequently prime CD4+ T cells to secrete IFN-g or on T cells. Such PSA-TLR2 signalling on T cells directs development of inducible Tregs by promoting Foxp3 expression and subsequent IL-10, TGF-B secretion (Round et al., 2011). Tregs than work in favour of B. fragilis and supress the activity of Th17 cells as well as IL-17 and IL-6 expression. This leads to suppression of intestinal inflammation and subsequent increase of tissue associated colonisation. However anti-inflammatory properties have been proven to be systemic and not only intestine located. Tolerogenic pDCs are present in cervical lymph nodes where they induce

IL-10 producing Tregs. These Tregs prevent diseases like experimental autoimmune encephalopatis (an animal model of multiple sclerosis) (Dasgupta et al., 2014).

PSA stimulation is also essential for balance between Th1 and Th2 response. It reduces IL-4 leading to decrease of Th2 activity. Conversely, PSA stimulates specific increase of Th1 cytokine production such as mentioned IFN-γ and IL-2. These are proinflammatory cytokines are important in antiviral and antitumor response. Even though their overproduction may lead to autoimmune disease, their adequate production is essential in systemic balance of Th1 and Th2. This equilibrium and adequate choice of response is fundamental for human immune development and function. Mice with absent PSA production showed thymic pathology. Their disease involved increased Th2 cytokine production which led to outgrowth of B cell follicles in thymic medulla. This resulted in numerous autoimmune diseases such as myasthenia gravis, a disease with impaired function of neuromuscular junctions (Mazmanian, Cui, Tzianabos, & Kasper, 2005).

3.4 Immunomodulatory protein

Recent finding describe bacterial molecule which is not a metabolic by-product of an energy-releasing process nor a structural component. It is a 15 kDa protein synthesized and secreted by commensal bacteria. It has been termed microbial anti-inflammatory molecule (MAM) because of its immunomodulatory effects. MAM is secreted by *Faecalibacterium prausnitzii* which belongs to *Firmicutes* phyla and *Clostridia* class. *F. frausnitzii´s* anti-inflammatory properties have been known over a decade however since *F. prausnitzii* is a high producer of butyrate it´s immunosuppressive mechanism could have been misinterpreted. MAM blocks NF-κB activation and IL-8 production by transfected IECs in vitro. However its effects were clear in treatment of inflammatory diseases in mice studies (Quévrain et al., 2016; Sokol et al., 2008).

Microbiota keeps a rich communication with its host to keep mutualistic character of symbiosis. This communication is crucial for host´s health and for microbial colonisation. It is mediated via number of modulatory molecules. However many pathways of communication with microbiota are still unknown. If the communication is disrupted host could develop diseases.

4 Dysbiosis and diseases

After previous chapter it is not hard to imagine that alteration of microbiota composition could change or abrogate the influential pathways of modulatory molecules. Such alteration could lead to differed immune system activity. Microbiota has thus role in many diseases. An alteration of microbiota associated with disease is termed Dysbiosis. Disease studies are good example of systemic microbiota relations. In this chapter will be described mechanisms of microbial involvement in several selected diseases.

4.1 Inflammatory bowel disease

4.1.1 Pathophysiology

Inflammatory bowel disease (IBD) is a disease characterised by chronic immune mediated intestine inflammation. Reasons of IBD occurrence are genetic predisposition and environmental factors. Several of such environmental factors modulate heavily microbial composition as well. Therefor has IBD been linked to microbiota dysbalance. The incidence of IBD is increasing world-wide suggesting an industrialisation relation. IBD is divided into two subtypes ulcerative colitis (UC) and Crohn´s disease (CD). UC and CD differ in pathopsysiology and manifestations but cellular principles remain the same (Ananthakrishnan, Khalili, Koinjeti, Hihuchi, & Silva, 2013; Molodecky et al., 2012).

Ulcerative colitis affects the colon, mainly rectal and sigmoid colon. In more advanced states the inflammation dilates to more proximal parts of the colon. These states are referred as left-sided colitis or more advanced extensive colitis which affects even transverse colon. The inflammation occurs mainly on mucosal layer with formation of specific structures pseudopolyps (regenerating IECs). Histological tests of the intestine show inflammation by granulocytes and mononuclear cells, distortion of glands and goblet cells depletion. Complications of ulcerative colitis include rupture of the bowel, massive bleeding, toxic megacolon and colon carcinoma. UC manifests mainly by blood and mucus in stool and abdominal pain. On the other hand Crohn´s disease affects mainly caecum and ileum of the small intestine. The inflammation often exceeds further to small intestine and forms skip lesions (skip areas), sections of inflammation separated by unaffected regions. Alternatively inflammation can exceed into large intestine where it creates isolated sections of inflammation. This stage is termed Crohn´s colitis. In CD the inflammation disrupts and affects all layers of the intestine. It is thus a transmural disease and often exceeds to serosa. Histological studies exhibit ulcerations over Peyers patches. Complications associated with CD are colon cancer, stenosis, fistula, abscess formation and granuloma. Manifestations are nausea, emesis, epigastric pain, dysphagia and diarrhea (Dejaco et al., 2003).

The cellular mechanism studied on mice involves signalling on DCs via TLR and NLR. DCs and macrophages secrete IL-23 which with influence of IL-6, IL-1β and TGF-β leads to expansion of Th17 cells and type 3 innate lymphoid cells. Actions also involve reactive oxygen species generation. The expansion of Th is followed by IL-17, IL-21, IL-22 secretion as well as Th1 activation. Th1s produce IFN-γ which stimulates reactive oxygen species (ROS) production by local innate immune cells. All these components collectively induce local inflammation and supress differentiation of Foxp3+ IL-10 secreting Tregs (Ahern et al., 2010; Feng et al., 2011). Chronic inflammation often causes epithelial damage which enables bacterial translocation and enhances the inflammation to systemic level.

4.1.2 Progression of disease

Factors which effect IBD occurrence and simultaneously microbial composition are usage of antibiotics, diet, faecal diversion treatment and (Shaw, Blanchard, & Bernstein, 2010). Dysbiosis is a characteristic of IBD patients. IBD patients show decreased microbial diversity and decrease of some probiotic species abundance (Takahashi et al., 2016). Relative abundance of *Firmicutes* is decreased whereas *Bacteroides* abundance is increased. The main question remains if the alteration of microbial composition is a cause or an effect. The answer to this question could be likely both. Even newly diagnosed patients with early stages of the disease show dysbiosis. However dysbiosis is amplified by the environmental changes caused by the inflammation. Since inflammation is an oxidative process, more aerobic species are able to utilise their metabolism and overgrow (Lopez et al., 2016). The possible principle could thus be following. An environmental factor alters the microbial composition. This altered composition possibly decreases number of probiotic species such as *B. fragillis*, cellulolytic species or *Fecalibacterium prausnitzii* and increases number of potentially pathogenic species (Sokol et al., 2008). These events could set off inflammation because of reduced tolerogenic stimuli by probiotic modulatory molecules and increased toxic stimuli by potentially pathogenic species (Takahashi et al., 2016). If the triggering factor remains present inflammation changes environmental conditions of the intestine which leads to long-term different species preference. This could be followed by epithelial damage and bacterial translocation. If there is bacterial translocation, tolerogenic systems fail. Bacteria and their antigens like LPS come to contact with innate and adaptive immune cells in the subepithelium. Pattern recognition receptors of innate immune cells of lamina propria bind according MAMPs which leads to proinflammatory cytokine secretion and enhanced immune cell recruitment. LP DCs maturate and migrate to MLNs to prime T and B lymphocytes. T lymphocytes than migrate towards inflammation and initiate protective and cytoxic activities. B lymphocytes maturate into plasmocytes and secrete antimicrobial antibodies (Xiong et al., 2014). These actions happen because the intestinal barrier has been damaged and immune system tries to prevent further systemic infection.

4.1.3 Probiotic treatment

Treatment of IBD with probiotics has been successful in numerous studies. Genetically modified bacterium which encoded MAM protein exhibited preventing effects of inflammatory bowel disease in mice studies. Preventing effects included lower production of IL-17 and IFN-γ cytokines as well as reduced weight loss in DNBS-induced colitis (Quévrain et al., 2016; Sokol et al., 2008). Interestingly effective IBD treatment was achieved by orally administered Lactobacillus casei lysate. This bacterial lysate provided prevention against increased intestinal permeability and barrier dysfunction. It also increased number of FoxP3+ regulatory T cells in MLNs and decreased LPS-induced production of cytokines TNF-α, IFN-γ and conversely even IL-10 (Zakostelska et al., 2011).

4.2 Colorectal cancer

Colorectal cancer is the third most common cancer. It has a 65 % five-year survival rate. As mentioned people with IBD have higher risk in developing colorectal cancer (CRC). In fact in animal models of CRC the disease is triggered by induction of inflammation and simultaneous stimulation with carcinogen. If the body is set in a chronic inflammation of IBD which is characterised by microbial dysbiosis it could cause following events. Dysbiosis decreases stimulation by probiotic modulatory molecules which monitor cell cycle of IECs (Singh et al., 2014). If epithelial damaged has yet not come, the inflammation weakens the intestinal barrier through decrease of supportive modulatory molecules and enables bacterial translocation. High inflammation state causes generation of ROS which damage DNA of epithelial cells causing possible transforming mutations. Inflammation is also associated with changes in bile acid metabolism to produce potentially carcinogenic secondary bile acid (Duboc et al., 2013). These events could lead to mutations and transformation of the cell and adenoma formation. Adenoma is a benig tumor. Benig tumors are usually relatively localised, capsulized and slow-growing. 5 % of adenomas could lead to formation of malignant lesion carcinoma (Carvalho et al., 2012). Carcinomas grow rapidly, they are uncapsulised and affect different cell types which makes them invade surrounding tissues. This phase inducts rich blood vessels supply development to enable the tumor growth. This makes it possible for the cancerous cells to separate from the tumor, enter the blood vessels and create metastases by systemic distribution (Carvalho et al., 2012). Furthermore the cancer-associated intestinal environment has altered characteristic of accessible substrates, pH and changes in redox potential. This may boost the changes in microbiota composition (Hirayama et al., 2009).

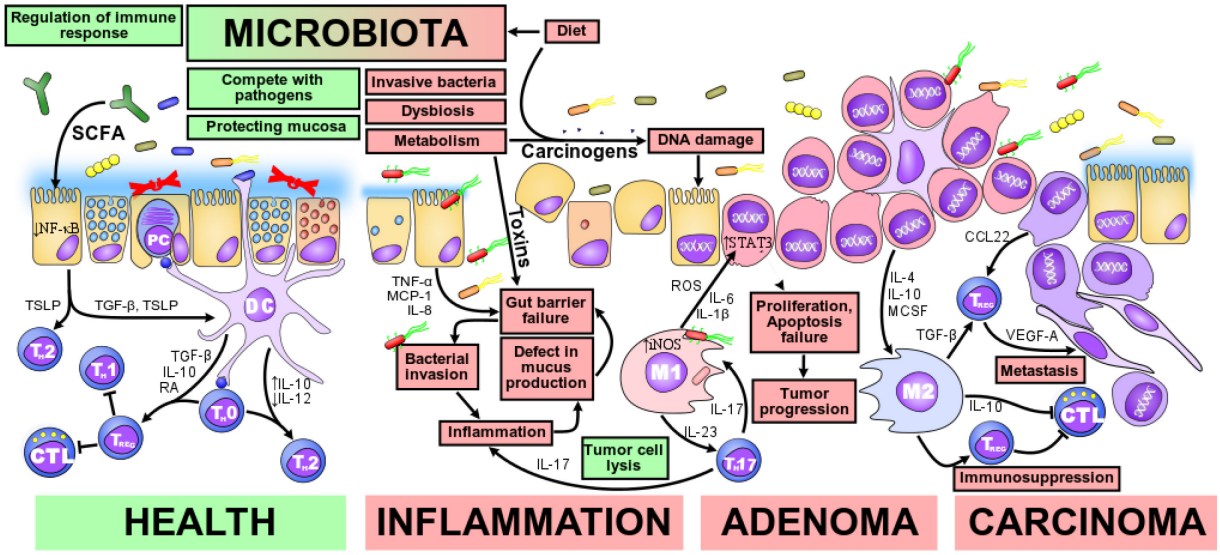

Figure 4: Gradual shift from health to carcinoma
Illustration provided by advisor MUDr. Miloslav Kverka Ph.D.

4.3 Allergy and asthma

A good example of failed tolerogenic functions is allergy and asthma. Both diseases increased rapidly in last decades. Their patients suffer from overreaction to common nonvirulent antigen stimuli. Atopic sensitisation and asthma immunity background involves high activation of Th2 cells which induce B cells to secret IgE antibodies. IgE is than attached to mastocytes which after antigen stimulation induct histamine production. Histamin causes bronchi contraction and increases blood vessel permeability which leads to asthma, edema and hives. Allergic children exhibit altered microbiota composition in terms of relative higher aerobic bacteria abundance. Th2 overproduction could be linked to dysbiosis since Th1 and Th2 balance is maintained by commensal bacteria as previously mentioned (Mazmanian et al., 2005). Furthermore several probiotics show positive effect in human allergy and asthma treatment. Infants colonised with several Lactobacillus and Bifidobacterium species in first week of life have lower chance in developing allergy (M. A. Johansson, Sjögren, Persson, Nilsson, & Sverremark-Ekström, 2011). Treatment of food-allergic infants with *Lactobacillus GG* helped to decrease atopic dermatitis. This is an example of treatment which affects tissues outside of digestive tract by modulation of intestinal microbiota composition (Viljanen et al., 2005).

4.4 Irritable bowel syndrome

Irritable bowel syndrome is a disease, which affects the intestine. However IBS is not defined by damage of the intestine or inflammation (although inflammation might occur before the disease or after). The manifestations include abdominal pain, constipation and/or diarrhoea. Association with microbiota was suggested because patients mentioned that disease manifested after an infectious diarrhoea (which alters the microbiota composition) and/or previous antibiotic treatment. Consequent patient studies also revealed high number of intraepithelial lymphocytes (suggesting higher activation of IS) and depressions (suggesting microbiota´s neuromodulatory impairment), influence of diet and finally an altered composition of microbiota. The pathophysiology of IBS is poorly understood, however several principles have been described. Diarrhoea and constipation are caused by abnormal bowel motility. Abdominal pain is caused by visceral hypersensitivity (Lacy et al., 2016).

Abnormal bowel motility is a disorder of autonomic system regulation. Microbiota modulates intestinal motility via affecting migrating myoelectric complex (MMC) (Husebye, Hellström, & Midtvedt, 1994). MMC leads the crucial mechanical patter of small intestinal motility. This pattern is essential to clear stomach and the intestine of residential food, secretions and desquamated cells (Telford & Sarna, 1991). Disruption of MMC is associated with dysbiosis. Furthermore specific intestinal motility changes have been linked to specific bacteria. For instance *Bacteroides thetaiotaomicron* in mice studies increased expression of specific muscle genes which modulate intestinal motility and genes which modulate enteric nervous system (Hooper et al., 2001).

Escherichia coli Nissle enhanced colonic contractility by stimulation of smooth muscle cells in vitro conversely pathogenic E. coli strain impaired the contractility via LPS stimulation (Bär et al., 2009).

Visceral hypersensitivity causes low pain threshold level via abnormally strong response of intestinal sensory nerve endings to stimuli such as stretching of the intestine. These afferents lead signal through enteric nervous system, dorsal horns of spina up to cortex for pain perception. Error could appear at any level of this signalling pathway for instance in the central processing. IBS showed patients an aberrant brain activation in left prefrontal cortex (H. S. Silverman et al., 1997). Germ free mice colonised with microbiota of IBS patients lowered their pain threshold of colonic distention whereas mice colonised with healthy microbiota did not. Dysbiosis triggered by stress in mice studies showed increase of visceral pain. Dysbiosis triggered by stress and ATB treatment however showed decreased visceral pain (Aguilera et al., 2013). This ATB effect is advantageous in terms of pain reduction. However dysbiosis triggered by ATB and stress was even more intense than in stress only. The effects could thus likely manifest in different dysregulation and ATBs seemed to mask a serious dysbalance. Probiotic treatment with *Lactobacillus rauteri* live culture or even lysate prevented pain response and reduced dorsal root ganglion activity in rat brain-gut axis (Kamiya et al., 2006).

Mentioned selected disease examples show well the impact of microbial dysbiosis and the potential of probiotic treatment. There are other diseases like rheumatiod arthritis or diabetes mellitus associated with the microbiome. However microbiota has also a major neuromoduling impact in neurotransmitter production (which did not fall into the aim of this thesis). Thus it likely plays a major role in psychiatric diseases such as depression and anxiety (Kan et al., 2018; Larsen et al., 2010; Yuichi et al., 2016).

4.5 Context

Multiple studies associated mentioned diseases with specific bacterial strains. Adherent-anvasive *E. coli* and *Mycobacterium paratuberculosis* were suggested to cause crohn´s disease (Darfeuille-Michaud et al., 2018; Thayer, Coutu, Chiodini, Van Kruiningen, & Merkal, 1984). *Enterococcus faecalis* induced DNA damage of epithelial cells by reactive oxygen species production could lead to carcinogenesis. However studies of one isolated bacterial species cannot describe the complexed interactions in human intestine. Individually harmless species could induce inflammation in presence of commensals. *Segmented filamentous bacteria* (SFB) induce Th17 recruitment in presence of other commensals (Ivanov et al., 2009). The inflammation could be favourable in terms of supressing pathogenic bacteria however in inflammatory disorders further Th17 recruitment exacerbates the pathologic progress. Use of probiotics as a treatment has been shown to be effective in multiple diseases however the context dependency debases unicity of results. Possible solution of this problem could be treatment by bacterial lysates. Treatment by bacterial lysate could decrease the context dependency of a live organism.

5 Conclusion

Microbiota provides essential stimuli for host´s health. It communicates with its host via intestinal mucosa and gut associated lymphoid tissues. Bacteria are here sampled and correspondingly either tolerated or repressed according to their nature. Communication is mediated by several microbial modulatory molecules including short-chain fatty acids, lipopolysaccharide, virulent factors, polysaccharide A and immunomodulatory proteins. The immune reactivity toward these molecules has probably evolved as a protective measure to prevent over-reactivity to stimuli provided by trillions of microorganisms

LPSs are recognised by PRRs TLR2 and TLR4 and set-off signalling pathway which increases NF-κB activity and subsequent proinflammatory cytokine secretion. However the intensity of response differs according to the structure of LPS and corresponding receptor. Commensal microbiota lacking pathogenic adaptations triggers lower responses. TLR4 expression on intestinal cells is also down-regulated and LPSs are detoxified by alkaline phosphatase to prevent damage from intestinal microbes.

SCFAs in higher concentrations are inhibitors of HDACs. This inhibition is specific and leads to decreased expression of inflammatory genes in enterocytes as well as in macrophages. This decrease is mediated via either increased expression of inhibitory proteins or lowered activity of NF-κB. Furthermore HDAC inhibition blocks DCs differentiation and stimulates Treg production and effectiveness in suppressive activities by modulation of Foxp3 activity. However SCFAs also interact with widely expressed GPCRs 43, 41 and 109A. This signalling increases anti-inflammatory cytokine secretion by DCs and macrophages, leads to favoured IL-10-secreting Tregs priming, increases PGE_2 production by monocytes, decreases immune cells recruitment by down-regulated chemotactic protein and adhesion molecules production, fortifies intestinal barrier integrity and overall inhibits responsiveness of innate and adaptive immune cells to LPS.

Component of *B. fragilis* capsule polysaccharide A is a presentable antigen and a ligand of TLR2. Its presentation on DCs collectively with TLR2 stimulation induces clonal expansion of Foxp3+ Tregs, stimulates IL-10 secretion and interestingly event Th1 cytokine secretion. These effects supress Th17 activity and balance Th1/Th2 response. Other commensal *Faecalibacterium prausnitzii* secretes an immunomodulatory protein termed MAM. MAM blocks NF-κB activation and IL-8 production by IECs.

Dysbiosis of microbial composition leads to disrupted communication and diseases like IBD, CRC, IBS, allergies and others. Most of these diseases feature an over-reactive immune system. Stimulation with probiotics, lysates or even separate suppressive modulatory molecules proved to be beneficial locally but also systematically outside of the intestine in treatment of diseases. The importance of

microbiota in physiology modulation and potential of probiotic treatment have been studied extensively in the last decade. Empiric knowledge is increasing however molecular background of specific processes is lacking. Microbiota is thus a very important field of study with substantive potential.

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